Transverse Magnetic Tweezers Allowing Coincident Epifluorescence Microscopy on Horizontally Extended DNA

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Abstract

Longitudinal magnetic tweezers (L-MT) have seen wide-scale adoption as the tool-of-choice for stretching and twisting a single DNA molecule. They are also used to probe topological changes in DNA as a result of protein binding and enzymatic activity. However, in the longitudinal configuration, the DNA molecule is extended perpendicular to the imaging plane. As a result, it is only possible to infer biological activity from the motion of the tethered superparamagnetic microsphere. Described here is a "transverse" magnetic tweezers (T-MT) geometry featuring simultaneous control of DNA extension and spatially coincident video-rate epifluorescence imaging. Unlike in L-MT, DNA tethers in T-MT are extended parallel to the imaging plane between ~~2~~two ~~µm~~micron-sized spheres, and importantly protein targets on the DNA can be localized using fluorescent nanoparticles. The T-MT can manipulate a long DNA construct at molecular extensions approaching the contour length defined by B-DNA helical geometry, and the measured entropic elasticity agrees with the worm-like chain model (force < 35 pN). By incorporating a torsionally constrained DNA tether, the T-MT would allow both the relative extension and twist of the tether to be manipulated, while viewing far-red emitting fluorophore-labeled targets. This T-MT design has the potential to enable the study of DNA binding and remodeling processes under conditions of constant force and defined torsional stress.

Keywords

Transverse magnetic tweezers
Coincident fluorescence microscopy
DNA micromanipulation
Single-molecule manipulation

1. Introduction

Magnetic tweezers (MT) have become a common single-molecule manipulation technique and are widely used to probe the elasticity of supercoiled DNA and the dynamics of DNA processing enzymes involved in modulating chromosome architecture [1]. Most MT instruments are designed to stretch out a single tethered DNA molecule orthogonal to the microscope coverslip surface ("longitudinal" configuration). Positioning of fixed pole magnets above the surface causes a superparamagnetic microsphere (SP-MS) attached at the untethered end of the DNA molecule to move away from the coverslip surface, thus stretching out the DNA molecule. The force acting on the DNA molecule is altered as the vertical position of the magnets is changed. In this longitudinal MT (L-MT) geometry it is possible to negatively and positively supercoil the tether by rotating the magnets, if the DNA molecule is attached to the SP-MS and

positively supercoil the tether by rotating the magnets, if the DNA molecule is attached to the SP-MS and coverslip surfaces via both DNA strands, i.e., torsionally constrained [2]. The dynamics of DNA processing enzymes can be studied using L-MT; however, in these experiments enzymatic activity is inferred from changes in DNA tether length and/or linking number [3]. This results in all information on the location of the enzymatic event on the DNA tether being lost. In addition, L-MT experiments necessitate a long-lived DNA–enzyme complex, thus it is not feasible to study enzymes or proteins that associate with DNA but do not alter its topology, e.g., proteins undergoing 1D sliding on DNA.

Although the majority of magnetic tweezers systems adopt the longitudinal configuration there are a few systems designed for use in a horizontal or "transverse" configuration, i.e., transverse magnetic tweezers (T-MT). These systems utilize the same basic principle as L-MT, whereby a single tether is extended between a stationary surface and a SP-MS moving in response to an applied magnetic field. However, unlike the longitudinal configuration, the tether is extended in the focal plane of the objective lens. This affords the notable advantage of permitting real-time observation of events on the tethered substrate, as with laminar flow extension and optical tweezers manipulation of DNA, while maintaining the ability to introduce positive or negative twist into the DNA molecule.

Currently, no standard configuration for a T-MT microscope exists, with relatively few systems having thus far been published. One of the first examples was reported by Danilowicz et al., where DNA tethers were formed between a SP-MS (2.8 μm diameter) and the antibody-functionalized surface of a cylindrical capillary (330 μm diameter) [4]. This assembly was placed inside a square micro-cell (600 μm cross-section), which permitted fluidic sample delivery and buffer exchange. Force was applied using a stack of five permanent magnets (each $6.4 \times 6.4 \times 2.5$ mm^3) placed to one side of the micro-cell and the corresponding SP-MS response observed using a 10× objective lens (NA = 0.25) placed underneath the sample. Although not explicitly stated, the low resolving power of the optics indicates a long working distance and was likely a compromise designed to permit both wide-field imaging and close proximity of the magnet stack and sample. While this allowed forces up to 30 pN to be measured simultaneously for dozens of tethers, the low magnification of the microscope limited its use to the reported multiplex application.

The compromise of low magnification in favor of a higher applied force was reversed in a similar design reported by Graham et al. [5]. In this system, tethers were formed directly onto the micro-cell surface (1 mm cross-section; VitroCells; VitroCom) and extended at an acute angle relative to this surface. Fluorescence imaging was done through the bottom surface of the micro-cell using a 60× magnification oil-immersion objective lens (NA = 1.25, PlanApo; Olympus) and epi-illumination. The SP-MS (Dynabeads M-280; Invitrogen) was manipulated using a stack of four cubic NdFeB magnets (12.7 mm cross section) held perpendicular to the objective lens optical axis on the end of a micromanipulator. With this configuration, forces up to 3 pN were tested; higher applied forces may have been possible but this was not reported.

Using an electron-multiplying CCD camera, Graham et al. were able to observe DNA-binding by the proteins Fis, HU and NHP6A with a high signal-to-noise ratio [5]; however, epi-illumination ultimately limits the contrast possible through significant bulk fluorescence excitation. This can be addressed via implementation of total internal reflection fluorescence (TIRF) microscopy as demonstrated by Schwarz et al. [6]. Fundamentally, the microscope configuration is nearly identical to that reported by Graham et al., but with tethers formed from the lower surface, permitting TIRF) illumination. This is only a partial solution, because the finite SP-MS diameter will result in non-horizontal tether inclination and limit the amount of DNA within the evanescent field. For typical 1 μm diameter SP-MS, only one-fifth of the tether will be within the 100 nm penetration depth of the evanescent field. While the exponential field decay will likely result in observation beyond this range, a significant decrease in fluorescence intensity would be observed. This would make molecular tracking and stoichiometry of DNA-associated protein complexes difficult to quantify. Similar to the epifluorescence system, this method also suffers from limited force generation, with the highest reported value being 1.5 pN when using a single cubic permanent magnet ($5 \times 5 \times 1$ mm^3; Q-05-05-01-HN; Supermagnete).

A permanent magnet-based tweezers system has also been reported by van Loenhout et al. [7]. They used a standard longitudinal configuration to initially twist DNA, but with a second magnet to pull the coiled tether horizontally. This was used in conjunction with the fluorescent dye Cy3 to view plectoneme dynamics in DNA. While demonstrated using epifluorescence, such a configuration is not too dissimilar to that described by Schwarz et al. [6]. As with the methods of Graham et al. and Schwarz et al., lateral forces appear to be restricted, with magnitudes no greater than 3.2 pN reported.

An alternative approach to realization of a T-MT is to use electromagnets, whereby electromagnetic coils placed either side of the sample generate a relatively uniform magnetic field [8]. While this configuration was reported to yield forces of ~15 pN (using M280 SP-MS; Invitrogen), the use of a water-immersion objective necessitated a larger coil spacing, thus limiting forces to ~1.7 pN. Furthermore, resistive heating of the coils required implementation of an active water-cooling system; a problem characteristic of electromagnets [8, 9]. Through implementation of micro-fabricated electromagnets, Chiou et al. were able to achieve three-dimensional control of magnetic substrates [10]. This configuration was reported to benefit from reduced heat generation and produce applied forces exceeding 20 pN when acting on 2.8 μm diameter SP-MS, while maintaining compatibility with high numerical aperture light microscopy and epi-illumination [10]. The notable disadvantage of such electromagnetic approaches is a significant increase in implementation complexity relative to a permanent magnet-based tweezers system~~s~~.

Several methods to manipulate individual DNA molecule extension while permitting simultaneous single-molecule fluorescence observation have been reported. Despite this, there is no easily applicable, standardized approach for manipulating twist in a horizontal DNA template. The emerging trend is to adapt the established L-MT technique, in which DNA is extended orthogonally from the tethering substrate, to permit extension within the observable plane of the microscope. However, the relatively few published techniques are limited in at least one of the following: maximum achievable applied force, optical spatial resolution and the capacity to generate truly horizontal tethers. Compromise between force and resolution is necessary since short sample-to-magnet separations are required to apply high force, yet such positioning is generally precluded by the large objective lenses used for high numerical aperture microscopy. Similarly, high spatial resolution and truly horizontal tethers have thus far been mutually exclusive, with inclined extension from the lower sample surface used in conjunction with TIRF microscopy.

There is a need for an easily implementable approach to allow single-molecule localization experiments to be conducted on torsionally constrained and characterizable DNA tethers. This can be realized through implementation of design alterations to the aforementioned horizontal magnetic tweezers configurations. Firstly, use of thin fluid cells (<10 μm) in which experiments are conducted limits bulk fluorescence excitation, thus facilitating use of epi-illumination, as opposed to spatially restricted TIRF illumination. As a result, tethers can be extended horizontally in the center of the sample chamber, rather than attached to the lower surface and extended at an acute angle. Secondly, use of a long-working distance objective lens to permit reduced sample-to-magnet separations increases the applicable force range dramatically. Finally, use of nanoscopic fluorescent probes (e.g., TransFluoSpheres; Invitrogen), rather than individual extrinsic organic dyes, reduces the deleterious effects sample photobleaching can have on the length of time over which DNA-associated events can be tracked. Additionally, the relatively large number of fluorophores present in a single TransFluoSphere further facilitates implementation of epifluorescence imaging, where reductions in signal-to-noise ratio relative to TIRF microscopy are inevitable.

In this chapter, a T-MT instrument is described which incorporates permanent magnets and a long-working distance microscope objective (Fig. 1). This enables the permanent magnet–superparamagnetic microsphere distance to be minimized, thus increasing the maximum force that can be applied to the DNA tether. This allows a single DNA tether to be manipulated at extensions equal to the molecular contour length determined by the B-form helix (=0.338 nm rise per base pair multiplied by number of base pairs in the DNA tether), where intramolecular interactions would occur with a very low probability (probability of loop formation in stretched polymer chain estimated in ref. 11) and protein-mediated

DNA looping could be probed. This design enables the entire length of the tether to be imaged using wide-field epifluorescence microscopy at video rate (30 Hz) (Fig. 2) in an easily constructed, thin (~9 μm) sample chamber (Fig. 3). This will allow fluorescently labeled targets to be visualized and tracked on the DNA tether as a function of DNA extension, i.e., applied force. By tracking the bright-field image of the SP-MS at a high sampling frequency (≥60 Hz), the variance of the SP-MS excursions in the lateral direction could be measured and, with the equipartition theorem, used to estimate the applied force on the DNA molecule (Fig. 4a) [2, 12].

Fig. 1

Schematic diagram showing tethering configuration for generating horizontal DNA tethers with a defined orientation. A single DNA tether is attached at one end to a 9 μm diameter ~~anti-digoxigenin IgG:Protein A/G~~Protein A/G:anti-digoxigenin IgG functionalized (PAG-AD) microsphere and at the other end to a streptavidin-functionalized superparamagnetic microsphere (SP-MS). The tether is extended horizontally using the force exerted on the superparamagnetic microsphere by a pair of permanent magnets. Fluorescently labeled DNA-bound protein could be observed from below using a long working-distance objective lens and wide-field epifluorescence microscopy

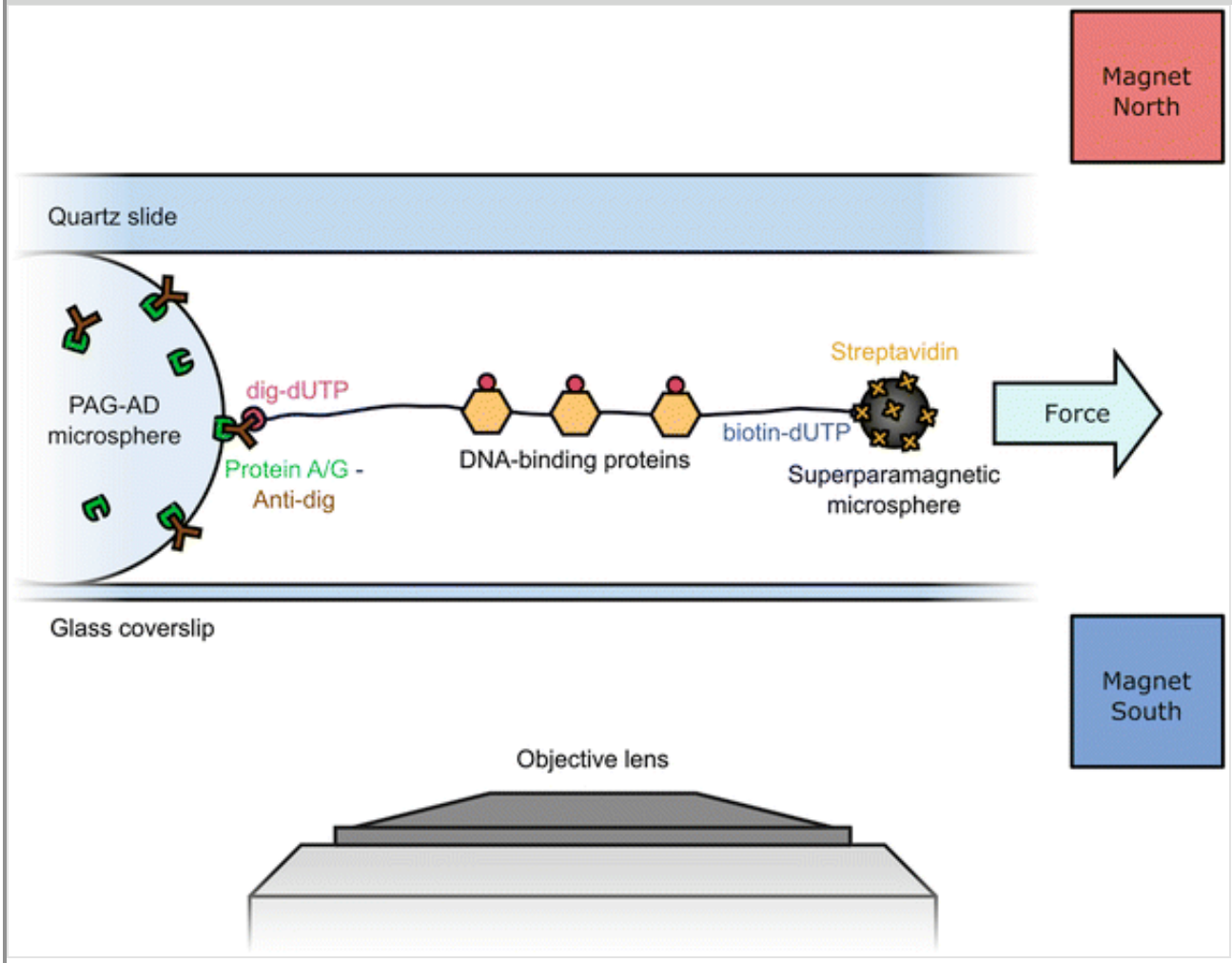


Fig. 2

(**a**) Optical configuration of the ~~"longitudinal~~transverse~~"~~ magnetic tweezers epifluorescence microscope. Bright-field illumination from a blue LED passes through the laser-coupling dichroic and is focused onto the CMOS camera by the tube lens. The excitation laser beam is expanded 4× using a Galilean beam expander and focussed to the back of the objective lens, whereby it excites fluorophores in the sample. Fluorescence emission follows the same path as transmitted bright-field light, but it is chromatically

Fluorescence emission follows the same path as transmitted bright field light, but it is chromatically separated and focussed onto an intensified CCD camera. (**b**) Exploded diagram of sample stage and components for sample and magnet-pair spatial control. Samples are held above the objective lens on a custom-fabricated stage with a commercially purchased micromanipulator for accurate sample translational control. A section cut from the stage allows the magnet pair to be brought into close proximity of the sample. The magnet pair is held in a clamp on the end of a rod connected to a stepper-motor-controlled rotational stage and the entire magnet-control assembly is placed on a one-dimensional micrometer-controlled translational stage, permitting precise movement of the magnets towards the sample. Since magnets are held with friction, magnet-pair separation can be easily adjusted if required

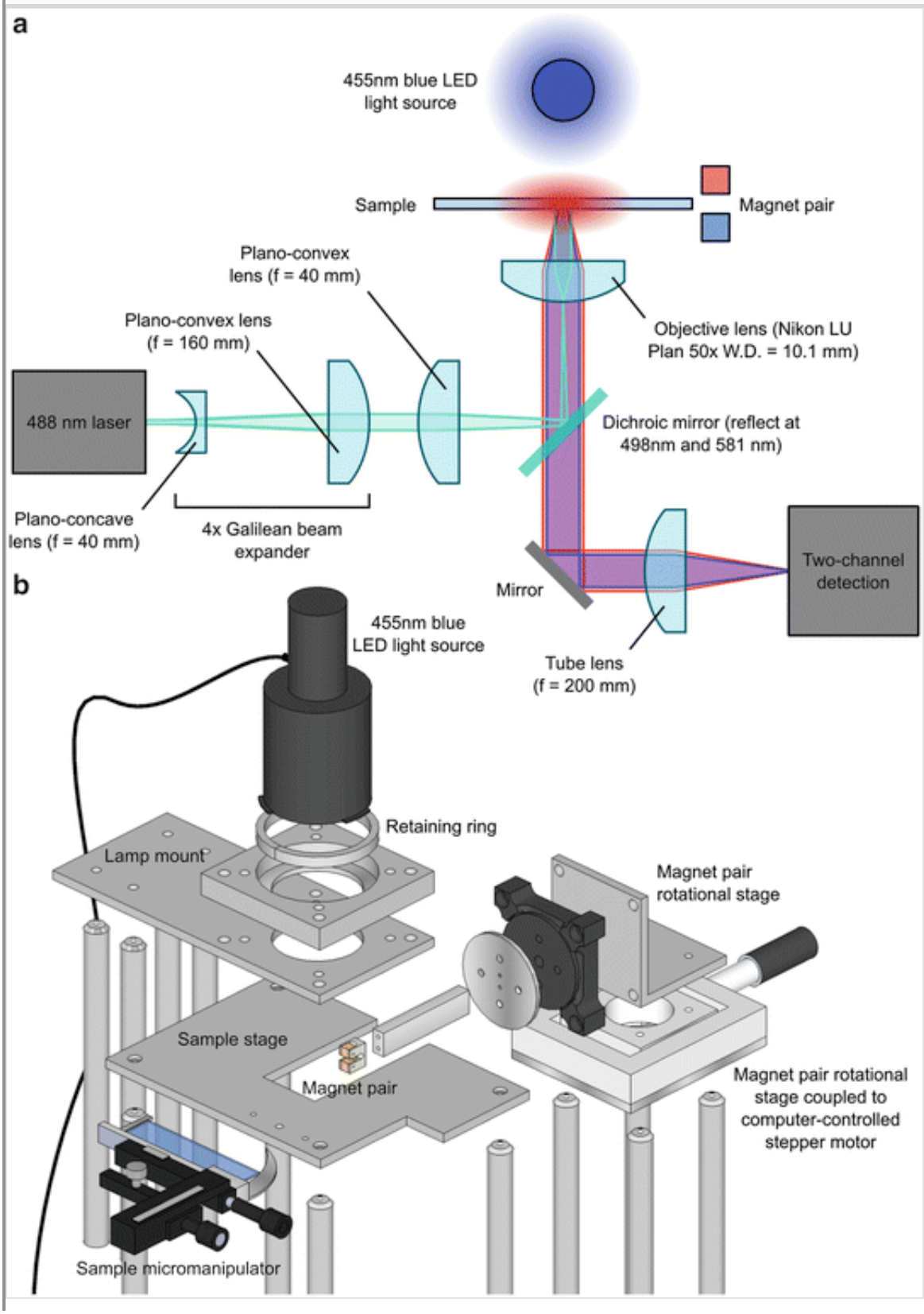


Fig. 3

Sample chamber preparation protocol for ~~longitudinal~~transverse magnetic tweezers microscope. (**a**) 10 μl of PAG-AD-MS mixture is deposited on a chemically cleaned quartz slide and trapped beneath a glass coverslip. (**b**) Opposite edges of the channel are sealed with nail varnish to create a flow-cell. (**c**) ~50 μl DNA-^{M280}SP-MS mixture is introduced along one open side of the channel and drawn through using a piece of tissue paper applied to the opposite side. (**d**) Following introduction of all reagents, the chamber is sealed along the remaining edges with nail varnish

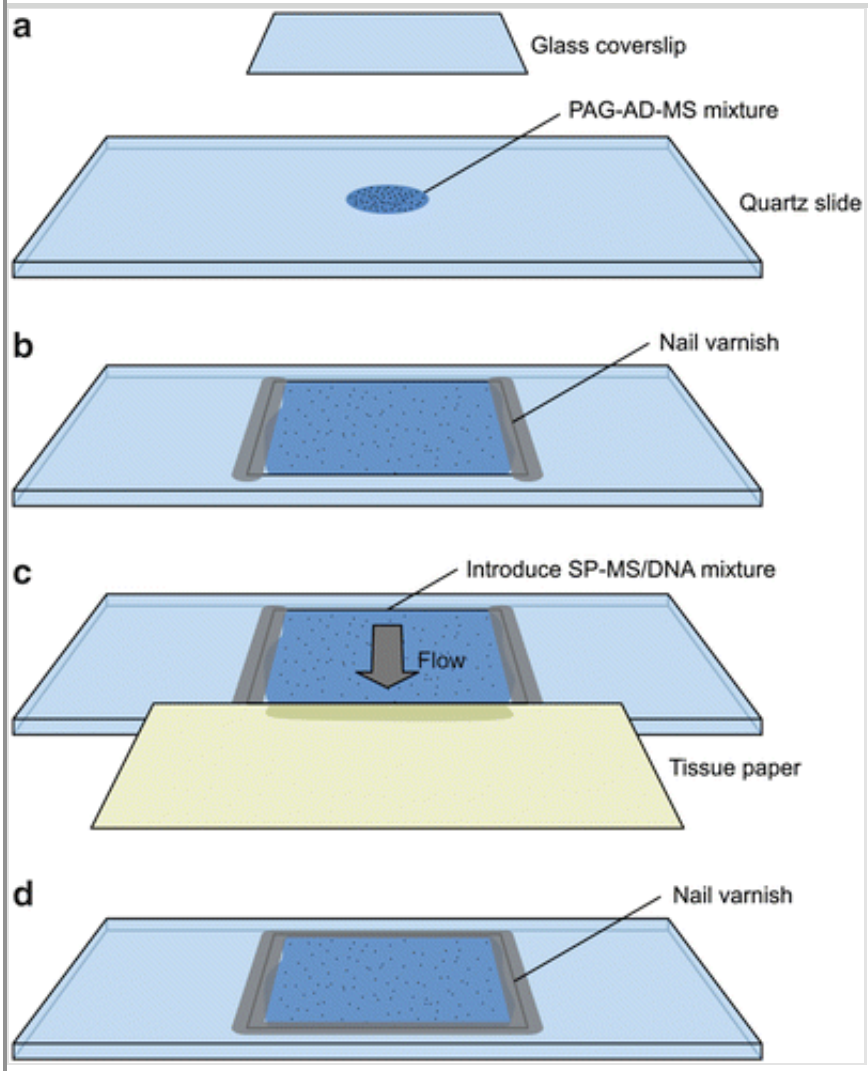
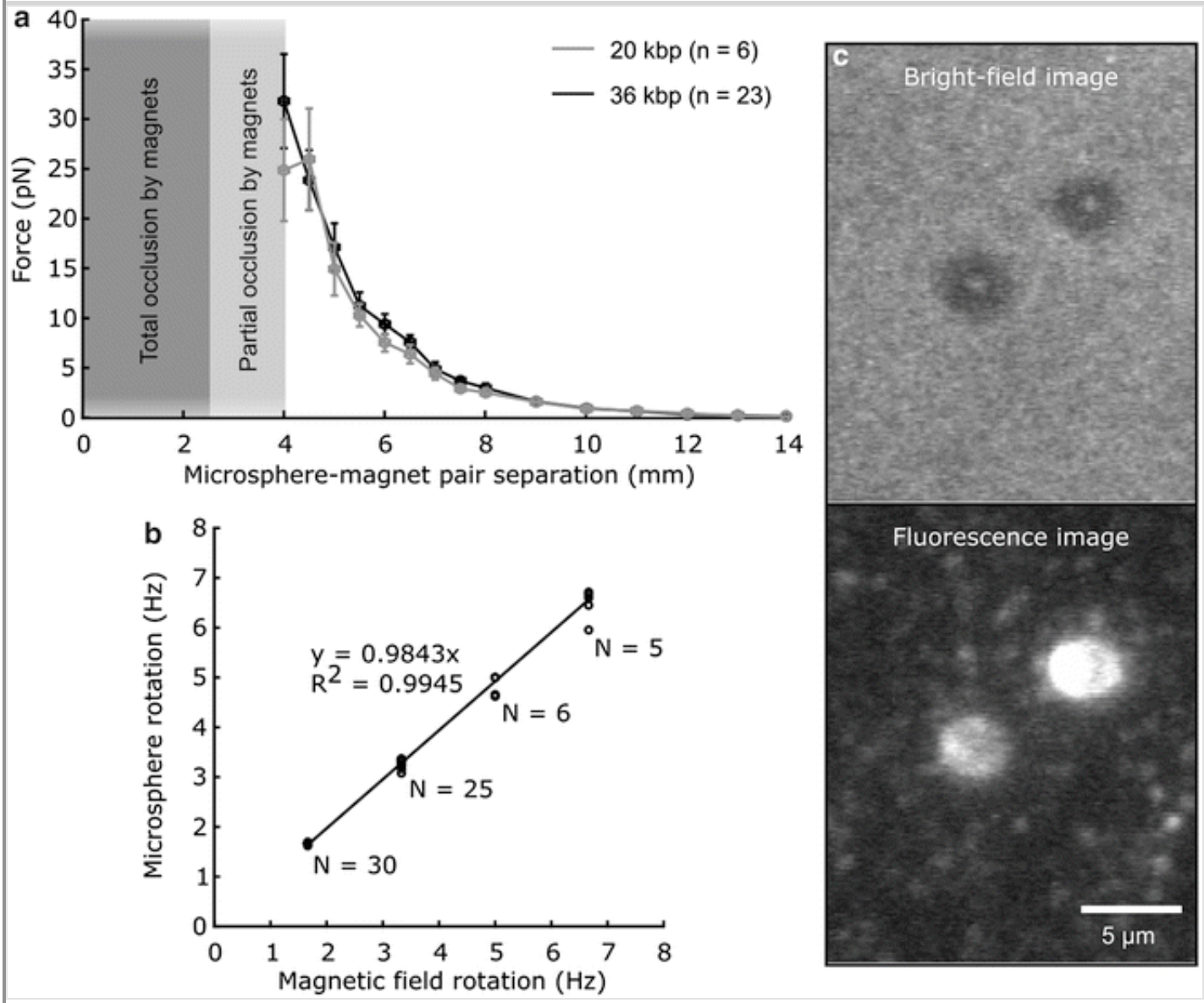


Fig. 4

(**a**) Force applied to DNA tethered (20,000 and 36,000 bp) superparamagnetic microspheres by an external magnetic field as measured through application of the equipartition theorem (*symbols*). This analysis treats DNA tethers as pendulums displaced from their equilibrium positions by thermal motion and relates the magnitude of the magnetic force acting on the tethers to the observed superparamagnetic microsphere lateral displacement sampled at a high video frame rate (60 fps, 1/200 s shutter time). (**b**) Rotation of DNA-tethered superparamagnetic microspheres. The permanent magnets were rotated using a computer-controlled stepper motor. Microsphere rotation was tracked in non-consecutive bright-field images (from video collected at 30 fps). Correlation between frequency of magnetic field rotation and the observed microsphere rotation was observed up to 6.7 Hz. Higher rotational frequencies were not possible with the ^{M280}SP-MS. (**c**) Simultaneous bright-field (*top*) and fluorescence (*bottom*) images from the ~~L~~T-

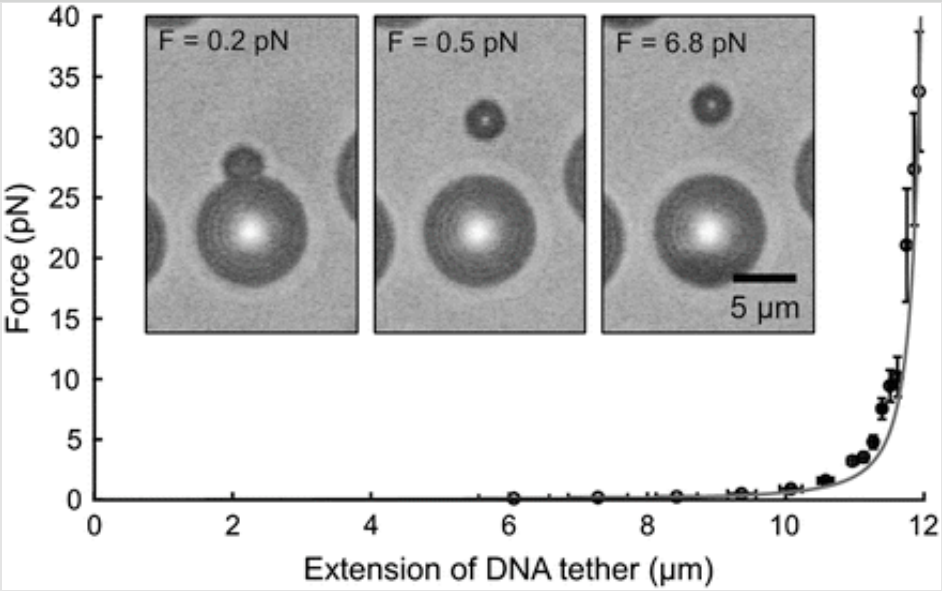
MT epifluorescence microscope. Sample containing ~0.1 nM TransFluoSpheres (diameter = 40 nm, λ_{ex} = 488 nm, λ_{em} = 655 nm) and ^{M280}SP-MS (diameter = 2.8 μm) was viewed using an intensified CCD camera. The ^{M280}SP-MS are intrinsically fluorescent, thus a narrow (655/20) band-pass filter must be used to select the TransFluoSphere fluorescence emission



The extension of the horizontal DNA was determined directly by measuring the distance between the pedestal microsphere (9 μm diameter, functionalized with Protein A/G:anti-digoxigenin IgG) and the superparamagnetic microsphere (^{M280}SP-MS functionalized with streptavidin) (Fig. 1), then subtracting the microsphere radii. The entropic elastic response of long DNA tethers (6000–36,000 base pairs, B-form contour length ≈ 2–12 μm) was measured (i.e., applied force versus extension of DNA tether, Fig. 5), and it compared favorably to the predictions of the worm-like chain model for a persistence length of 53 nm [13, 14]. In addition, we show that SP-MS can be spun at up to 6.7 Hz (Fig. 4b) by rotating the magnetic field on an axis coincident with the helical axis of the extended DNA tether. If a torsionally constrained DNA tether were utilized [2, 15], then positive or negative twist could be introduced at a constant elongational force to generate DNA supercoils. Simultaneous observation of the DNA tether by epifluorescence microscopy is possible and individual 40 nm diameter TransFluoSpheres can be detected (Fig. 4c). This enables fluorescently labeled proteins to be tracked and their stoichiometry to be quantified while the degree of DNA supercoiling is monitored or actively manipulated in real-time.

Fig. 5

Plot of measured force as a function of DNA tether extension. A small initial force (<1 pN) is required to achieve a relative extension of 0.8; however, the force necessary to continue extension increases rapidly beyond this point. This entropic elastic response is in good agreement with the theoretical, worm-like chain model (36,000 bp DNA contour length = 12.2 μm, persistence length = 53 nm). Inset bright-field images: Video frames taken from a ~~longitudinal~~transverse magnetic tweezers experiment, where increasing force applied by the magnet pair (relatively positioned at the *top* of the image) pulls the smaller superparamagnetic microsphere away from the larger, stationary microsphere (*bottom* of image). Agreement here further demonstrates the successful and reliable manipulation of a single DNA molecule



2. Materials

2.1. Buffers and Special Reagents

1. 25 mM MES buffer: Dissolve 1.22 g anhydrous 2-[*N*-morpholino]ethanesulfonic acid (MES, Sigma) in water (heating to 50 °C will aid dissolution) and add 31 ml of 0.1 M NaOH. Allow solution to cool and then increase the volume to 250 ml with water and check pH = 6.

2. Phosphate-buffered saline (PBS): Add 8 g NaCl, 1.44 g Na_2NPO_4, 0.24 g KH_2PO_4, and 0.2 g KCl to 800 ml of ultrapure water (resistivity of 18.2 MΩ cm). Adjust pH to 7.4 and increase volume to 1 L, then sterilize by autoclaving or filtering.

3. Tris–EDTA (TE): 10 mM Tris–HCl and 1 mM EDTA. Adjust pH to 8.0.

4. BSA coat buffer (BCB): 10 mM Tris–HCl, 172 mM NaCl, 1 mM EDTA, and 1 mg/ml acetylated BSA. Adjust pH to 8.0.

5. Tethering buffer (TetB): 10 mM Tris–HCl, 172 mM NaCl, 1 mM EDTA, and 0.1 mg/ml acetylated BSA. Adjust pH to 8.0.

6. 20 mg/ml acetylated BSA (Sigma)

2.2. Preparation of Microspheres Labeled with Anti-digoxigenin (PAG-AD-MS)

1. 4 % (w/v) aldehyde-sulfate polystyrene-latex microspheres (~9 μm diameter, Molecular Probes).
2. BSA-passivated 1.5 ml microcentrifuge tubes (*see* **Note 1**).
3. 5 mg/ml Purified Recomb® Protein A/G from *E. coli* (Pierce).
4. 1 M glycine: Dissolved in PBS.
5. 5 mg/ml sheep anti-digoxigenin polyclonal IgG (AbD Serotec).

2.3. Preparation of Oxygen Scavenger System

1. Degassed buffer: Relevant buffer degassed under vacuum with stirring for at least 15 min. Degassed buffer is stored in a plastic syringe fitted with a 25-G needle and used immediately.
2. 1 M dithiothreitol (DTT, Melford).
3. 300 mg/ml glucose (Fisher Scientific).
4. 10 mg/ml glucose oxidase (Sigma) stored as single-use aliquots at –20 °C.
5. 2 mg/ml catalase (Sigma) stored as single-use aliquots at –20 °C.
6. 1 ml plastic syringe fitted with a 25-G needle to minimize air-exposure of buffer

2.4. Preparation of Microscopy Substrates

1. Borosilicate glass coverslips (No. 1, 22 mm × 64 mm; Menzel-Gläser).
2. Quartz slides (1 mm thick, 75 mm × 25 mm; UQG Optics Ltd.).
3. 2 % (v/v) Neutracon (Decon Laboratories Ltd.).

2.5. Preparation of Horizontal DNA Tethers

1. ~2 % (w/v) anti-digoxigenin-functionalized ~9 μm diameter polystyrene-latex microspheres (PAG-AD-MS).
2. Dynabeads® M280 streptavidin-labeled superparamagnetic microspheres (^{M280}SP-MS, 2.8 μm diameter; Invitrogen) (alternative SP-MS are also compatible, *see* **Note 2**).
3. Double-stranded DNA template differentially end-labeled with biotin and digoxigenin was prepared according to a published method [16].
4. TransFluoSphere (488/645) streptavidin-labeled microspheres (40 nm diameter; Invitrogen).
5. Cubic gold-plated NdFeB magnet (5 mm × 5 mm × 5 mm; Supermagnete).

6. Clear nail varnish.

7. Lint-free optical tissue (SPI Supplies, Structure Probe Inc.).

2.6. Combined Magnetic Tweezers and Epifluorescence Microscope

1. DNA is extended and torsionally constrained using a ~~longitudinal~~transverse ~~configuration~~ magnetic tweezers (~~L~~T-MT) setup. The microscope is entirely constructed using a 30 mm cage system (ThorLabs) as detailed elsewhere [17]. This configuration combines wide-field epi-illumination and bright-field illumination to permit simultaneous observation of fluorescently labeled DNA-bound enzymes and measurement of applied force (via equipartition analysis of SP-MS motion [12]).

2. Fluorescence excitation is provided by a diode laser ($\lambda_{ex} = 488$ nm, 75 mW; Coherent Sapphire) with the beam diameter expanded 4× using a lens pair (focal lengths = 40 and 160 mm, diameters = 16 and 25 mm, respectively; Comar) in Galilean beam expander configuration. The laser is focussed to the back aperture of the objective lens and is coupled into the optical path using a dichroic filter (reflection at $\lambda = 498$ and 581 nm, FF498/581; Semrock) (*see* **Note 3**).

3. Bright-field illumination is provided by a blue LED ($\lambda_{max} = 455$ nm; ThorLabs) and is isolated from the fluorescence signal using a second dichroic filter (590 DCXR; Optical Insights) held in a Dual-Cam™ image splitter (Optical Insights). This simultaneously projects two chromatically separated images onto separate cameras for fluorescence (HQ655/20 band pass, Chroma; IC-300B intensified CCD, Photon Technology International) and bright-field (DMK 22BUC03 CMOS; The Imaging Source GmBH) imaging.

4. A long working-distance objective lens (W.D. = 10.1 mm; N.A. = 0.55; CFI LU Plan EPI ELWD, Nikon) is used for sample imaging due to spatial compatibility with the cubic NdFeB magnets, which are placed between the sample and lens.

5. The NdFeB magnets are friction-clamped at the end of an aluminum arm, which is mounted on a rotational stage (*see* **Note 4**). This is mounted on a translational stage, allowing the magnet pair to be rotated next to the sample at a user-defined distance. The magnets are aligned in a parallel, but opposed biaxial configuration with a gap of 0.4 mm, corresponding to the minimum separation possible with the sample chamber able to move freely between them.

6. A modified microscope stage is used, which permits the magnet arm to travel laterally towards the sample, in the plane of the sample.

7. Forces are measured through application of the equipartition function, relating variance of lateral SP-MS excursions to the applied force [12].

3. Methods

3.1. Preparation of Microspheres Labeled with Anti-digoxigenin

1. The 9 μm diameter sulfate-aldehyde functionalized polystyrene-latex microspheres (MS) are washed prior to functionalization in the following manner: 0.6 ml of 4 % (w/v) MS is added to 0.6 ml of 25 mM MES buffer (pH 6.0) in a BSA-coated microcentrifuge tube (*see* **Note 1**) and

agitated by vortexing at 1200 rpm for 30 s. The suspension in then centrifuged (*see* **Note 5**) at 750 × *g* for 2 min to pellet the MS. The supernatant is removed and is immediately replaced by an equal volume of fresh 25 mM MES buffer (pH 6.0). This process is repeated twice more with the final resuspension in 1.2 ml 25 mM MES buffer (pH 6.0).

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2. Add 100 µl 5 mg/ml ~~p~~Protein A/G (final concentration is ~0.385 mg/ml) and incubate overnight at room temperature (~20 °C) on a vertically inclined rotating turntable (or mix in an equivalent gentle manner) to prevent PAG-MS sedimentation.

3. Following incubation, pellet the MS by centrifugation at 750 × *g* for 2 min, then remove the supernatant and resuspend in a 1.2 ml 1 M glycine. Incubate this solution at room temperature for 40 min on a vertically inclined rotating turntable (or mix in an equivalent gentle manner).

4. After incubation with glycine, vortex and centrifuge the MS as described in **step 1**, then remove the supernatant and resuspend in 1080 µl PBS (pH 7.4) and 120 µl 20 mg/ml acetylated BSA (add the PBS first). Repeat this wash step two further times with the final resuspension in 1074 µl PBS (pH 7.4), 120 µl 20 mg/ml acetylated BSA, and 6 µl 2 % (w/v) sodium azide.

5. Protein A/G MS (PAG-MS) can be stored for extended durations (up to 6 months) in a fresh BSA-passivated microcentrifuge tube at 4 °C until required.

6. Prior to conjugation of anti-digoxigenin IgG, 100 µl PAG-MS is added to 100 µl TetB in a fresh BSA-passivated 0.5 ml microcentrifuge tube. The PAG-MS are washed three times with 0.2 ml TetB using the vortex, centrifugation and resuspension protocol from **step 1**. Resuspend in 100 µl TetB after the final wash step.

7. Add 3.2 µl 5 mg/ml anti-digoxigenin IgG (final concentration is 0.16 mg/ml) to 100 µl PAG-MS solution and incubate at room temperature for 1 h on a vertically inclined rotating turntable (or mix in an equivalent gentle manner).

8. Following the incubation, the anti-digoxigenin functionalized PAG-MS (PAG-AD-MS) are washed three times with 0.2 ml TetB using the vortex, centrifugation, and resuspension protocol from **step 1**. Resuspend in 100 µl TetB after the final wash step.

9. Store the ~2 % (w/v) anti-digoxigenin functionalized PAG-MS (PAG-AD-MS) in a fresh BSA-passivated 0.5 ml microcentrifuge tube at 4 °C and use within 24 h (*see* **Note 6**).

3.2. Preparation of Oxygen Scavenger System

1. Mix 960 µl degassed experimental buffer (normally TetB), 20 µl 1 M DTT, 10 µl 300 mg/ml glucose, 5 µl 10 mg/ml glucose oxidase, and 5 µl 2 mg/ml catalase in a 1.5 ml microcentrifuge tube.

2. To minimize solution exposure to air, transfer to a 1 ml plastic syringe fitted with a 25-G needle.

3. Store oxygen scavenger solution on ice (or at 4 °C) and use within 24 h.

3.3. Preparation of Microscopy Substrates

1. Place glass coverslips in a rack inside a water bath-compatible container (*see* **Note 7**).

2. Add 2 % (v/v) Neutracon solution so that coverslips are completely submerged, then sonicate (*see* **Note 8**) coverslips at 50 °C for 10 min.

3. Following sonication remove rack from cleaning solution and rinse thoroughly with deionized water (18.2 MΩ cm), then blow dry with filtered compressed air. Store coverslips in a sealed dust-free container until required.

4. Repeat the process for quartz slides (*see* **Note 9**).

3.4. Preparation of Horizontal DNA Tethers

1. Prior to coupling to DNA, streptavidin-functionalized superparamagnetic M280 microspheres (^{M280}SP-MS) are washed using the following process: Add 50 µl of 1 % (w/v) ^{M280}SP-MS to a 0.5 ml microcentrifuge tube, then increase total volume to 80 µl by adding TetB and mix gently. Hold an NdFeB magnet directly next to the tube and allow ^{M280}SP-MS to collect on tube wall for 60 s. Gently remove supernatant while the magnet is still in contact with the tube, then remove the magnet and resuspend in 80 µl TetB. Vortex microspheres at 1200 rpm for 30 s to ensure complete resuspension of the ^{M280}SP-MS pellet. Repeat the process twice more, with the final resuspension in 79 µl TetB to yield a ~0.6 % (w/v) solution.

2. To tether DNA to ^{M280}SP-MS, add 1 µl 2.8 nM differentially end-labeled DNA (*see* **Note 10**) to the microcentrifuge tube and incubate at room temperature for 1 h on a vertically inclined rotating turntable (or mix in an equivalent gentle manner).

3. Following incubation, any uncoupled DNA is removed from solution using the washing process described in **step 1**, with each resuspension in 160 µl of TetB. Store the sample on ice (or at 4 °C) and use within 24 h.

4. Take a clean quartz slide and place a 10 µl drop of 2 % (w/v) PAG-AD-MS in the center (Fig. 3a). Carefully place a Neutracon-cleaned coverslip over the droplet (Fig. 3a). Through capillary action, the solution should distribute evenly beneath the coverslip.

5. Seal opposite edges of the coverslip to the slide surface using nail varnish (Fig. 3b) (*see* **Note 11**). Allow 10 min for the nail varnish to harden, ensuring the chamber does not dry out during this period via evaporation at the open edges. TetB can be pipetted (~10 µl) along the open edges to prevent this occurring.

6. Once the varnish is dry, 10 µl of the DNA-^{M280}SP-MS sample is pipetted along one of the open edges. A sheet of lint-free optical tissue is placed along the opposite open edge to pull the sample through the chamber (Fig. 3c). Continually replenish the DNA-^{M280}SP-MS until 50 µl of DNA-^{M280}SP-MS sample has passed through the chamber. This process can take up to 10 min (*see* **Note 12**).

7. Add 10 µl TransFluoSphere-labeled DNA-binding protein (incorporating a biotin-tag) containing solution with a pipette along the open edge and use a fresh lint-free optical tissue to pull the sample through the chamber (*see* **Note 13**). The final concentration of TransFluoSpheres should be <1 nM (in experimental buffer with oxygen scavenging system added) to reduce the background fluorescence. A higher concentration of fluorescently labeled DNA-binding protein can be loaded in the chamber to facilitate DNA association; however, the fluorophore-containing buffer in the chamber must be exchanged with fresh experimental buffer until the background

fluorescence is reduced enough to resolve single TransFluoSpheres (wash with 50–100 μl buffer, *see* **Note 14**).

8. Use another lint-free optical tissue to remove any excess liquid from the open edges, then seal with nail varnish (Fig. 3d). Wait for 10 min for nail varnish to dry before using sample.

3.5. Manipulation of DNA-Tethered Superparamagnetic Microspheres

1. To prevent premature DNA-shearing, retract the magnet pair to a minimum separation of 10 mm from the nearest sample edge. Rotate the magnets to the vertical position, such that the air gap between them is aligned with the sample (Fig. 2b); this will allow the magnets to pass around the sample when the sample-magnet separation is reduced.

2. Take the assembled sample (from Subheading 3.4) and place coverslip side down on the microscope stage. Clamp in place using the micromanipulator and focus the objective lens on the AD-MS and ^{M280}SP-MS in the chamber.
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3. Slowly move the magnet pair towards the sample until the ^{M280}SP-MS begin to move freely in response to the magnetic field. Leave sample to stand until no further ^{M280}SP-MS motion is observed. At this point, all remaining ^{M280}SP-MS in the sample volume should be tethered or nonspecifically immobilized on the chamber surface. Nonspecifically adsorbed ^{M280}SP-MS can be easily distinguished from DNA tethered ^{M280}SP-MS by their lack of thermally induced motion at low applied forces ($F < 2$ pN) (*see* **Note 15**).

4. Measurement of force acting on ^{M280}SP-MS requires acquisition of high-speed video (≥60 fps) of tethered ^{M280}SP-MS for at least 15 s (*see* **Note 16**). The ^{M280}SP-MS centroid is tracked using a particle-tracking algorithm (*see* **Note 17**) to obtain ^{M280}SP-MS xy-coordinates. The variance of the lateral ^{M280}SP-MS displacement (i.e., relative to helical axis of tethered DNA) is translated into applied force using the equipartition theorem [12].

5. The elastic properties of the DNA tether are characterized by obtaining force (F, measured as described above) versus extension (x) data. From this data, it is possible to determine the apparent contour length (L_o) and persistence length (L_p) of the tether, thus confirming whether a single or multiple DNA molecules are forming the tether. In order to obtain the F versus x data, the distance between the microspheres is increased or decreased incrementally by translating the magnet pair. The PAG-AD-MS to ^{M280}SP-MS distance is determined at discrete molecular extensions and used to obtain x for the DNA molecule after subtracting the microsphere radii. The inextensible worm-like chain model of DNA elasticity [13] is then used to relate F, x, L_o, L_p and thermal energy (kT) at the experimental temperature (T).

4. Notes

1. Add 100 μl 20 mg/ml acetylated BSA to 900 μl of PBS (pH 7.4) in the 1.5 ml microcentrifuge tube to be passivated. Attach tubes to a vertically inclined rotating turntable (or mix in an equivalent gentle manner) while incubating at room temperature (~20 °C) for 1 h. The BSA mixture is discarded and tubes are subsequently washed three times with 1 ml of ultra-pure water (18.2 MΩ cm). Tubes are stored at 4 °C until required.

2. In addition to ^{M280}SP-MS (Invitrogen), successful tether formation and manipulation was demonstrated using Dynabeads® MyOne streptavidin-labeled T1 superparamagnetic

demonstrated using Dynabeads® MyOne streptavidin labeled T1 superparamagnetic microspheres (1.05 μm diameter; Invitrogen).

3. Specified optical components are also compatible with replacement of 488 nm laser with one centered on 561 nm.

4. The stepper motor (Reliance Cool Muscle, Reliance Precision Limited) coupled to the rotational stage is remotely operated by custom software (5000 positions per revolution) created in Microsoft Visual Studio (Microsoft Corporation). Closed-loop vector drive control ensures motor positioning is ultra smooth.

5. A bench-top microcentrifuge can be used for this centrifugation step.

6. Once functionalized with anti-digoxigenin, microspheres should be used within 24 h. Degradation of functionalization will be evident as a decrease in the frequency of DNA tether formation.

7. Racks for sonication should allow coverslips/slides to be spaced at least 1 mm apart to ensure good access for the cleaning solution. Racks must fit into water bath-compatible containers, such that coverslips/slides can be completely submerged in cleaning solution.

8. A sonicating water bath (Ultrawave Ltd.) is used for cleaning coverslips/slides.

9. Quartz slides can be reused after cleaning. Soak slides overnight in acetone to remove nail varnish and coverslips. The slides are then cleaned as follows: 10 min sonication (*see* **Note 8**) in isopropanol, rinse with deionized water, 10 min sonication in 1 M KOH (no heating), rinse very well with deionized water, immerse in fresh absolute ethanol, transfer to clean slide holder and incubate at 70 °C until dry. Store clean slides in a sealed dust-free container until required.

10. The DNA concentration can be decreased to change the ratio of DNA to ^{M280}SP-MS and reduce the likelihood of multiple DNA tethers forming. For example, DNA concentrations of 3.4 and 10 pM correspond to a 5- and 15-fold excess relative to the ^{M280}SP-MS, respectively.

11. During coverslip placement and edge sealing, motion of the coverslip should be minimized. At 10 μl, the deposited sample droplet should be sufficiently small that the coverslip binds tightly to the slide. If this is not the case, reduce the deposited volume.

12. Progress of the DNA-^{M280}SP-MS sample through the chamber can be observed due to the dark brown color of the ^{M280}SP-MS. If the flow rate is too low, the chamber can be tilted slightly using a pipette tip under one edge. Continued failure to obtain buffer flow is likely an indication that a larger initial droplet of AD-MS is required.

13. Samples containing TransFluoSpheres should be handled in a darkened room in order to minimize fluorophore photobleaching.

14. This step can be omitted if TransFluoSphere labeling is not required. If the step is omitted, the chamber must be flushed with fresh experimental buffer (50–100 μl) to remove untethered SP-MS before proceeding to **step 8** of Subheading 3.4 .

15. The rate of nonspecific adsorption is minimized by inclusion of BSA in TetB, which passivates the surface during **step 6** of Subheading 3.4 . If excessive adsorption is observed, the chamber can be washed with BCB (50–100 μl) prior to addition of the DNA-^{M280}SP-MS sample.

16. Image acquisition rate must be shorter than the characteristic relaxation time of the system (τ_0) [18].

[18]. Acquisition times longer than this will result in blurring of the microsphere image and a perceived reduction in amplitude of oscillation.

17. In each video frame, a small region of interest (ROI) is isolated round the SP-MS to be tracked. This ROI is rotated 180° and spatially shifted relative to the non-rotated ROI using image registration-based cross-correlation (*imregister.m* function; MATLAB, MathWorks) [19]. Localized SP-MS positions in adjacent frames are linked using a nearest-neighbor approach (up to user-defined spatial and temporal thresholds).

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